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Composition and Nature of Heterochromatin in the Electrical Fish (Knifefishes) *Gymnotus* (Gymnotiformes: Gymnotidae)

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Abstract

Fishes of the genus *Gymnotus* have been suggested as a good model for biogeographic studies in the South American continent. In relation to heterochromatin, species of this genus have blocks preferably distributed in the centromeric region. The content of these regions has been shown to be variable, with description of transposable elements, pseudogenes of 5S rDNA and satellite sequences. In *G. carapo* Clade, although geographically separated, species with $2n = 54$ chromosomes share the distribution of many 5S rDNA sites, a unique case within the genus. Here, repetitive DNA sequences from *G. sylvius* ($2n = 40$) and *G. paraguensis* ($2n = 54$) were isolated and mapped to understand their constitution. The chromosome mapping by FISH showed an exclusive association in the centromeres of all chromosomes. However, the cross-FISH did not show positive signs of interspecific hybridization, indicating high levels of heterochromatic sequence specificity. In addition, COI-1 sequences were analyzed in some species of *Gymnotus*, which revealed a close relationship between species of clade $2n = 54$, which have multiple 5S rDNA sites. Possibly, the insertion of retroelements or pseudogenization and dispersion of this sequence occurred before the geographic dispersion of the ancestor of this clade from the Amazon region to the hydrographic systems of Paraná-Paraguay, a synapomorphy for the group.

Keywords: FISH, Biogeography, Satellite DNA, rDNA 5S, *Cot-1*

1. Introduction

Repetitive DNA sequences are broadly distributed in eukaryotes genomes [1] and are classified into two categories: 1) repetitive sequences arranged in tandem as satellite, minisatellite, or microsatellite DNAs composed of hundreds of base pairs repeated a thousand times or more in each genome; and 2) moderately to highly repetitive sequences spread throughout the genome as retroelements or transposable mobile elements [2].

Copies of repetitive sequences are commonly associated with heterochromatin regions that can be visualized by C banding. These sequences are extremely

important to the functional and structural organization of the eukaryote genome, composing, for example, the pericentromeric heterochromatin regions [3, 4]. The heterochromatin in fish chromosomes is largely located in pericentromeric regions and has structural functions [5–7].

The studies about the location of repetitive sequences on chromosomes has broadened the knowledge of the structural organization of chromatin in fish, revealing an association of ribosomal DNA, telomeric sequences, transposition elements and satellite sequences in chromosomal rearrangements and weak break points [8, 9], in the fixation of sex chromosomes [10, 11], in the expansion of heterochromatin [12, 13] and in gene regulation [14]. This advances in molecular cytogenetics have demonstrated that repetitive DNA sequences are useful as chromosomal markers in studies of species evolution and can provided valuable information about sex chromosome systems and chromosomal rearrangements [15]. The mapping of Non-long terminal repeat (non-LTR) retrotransposable elements, the Rex in the fish species, for example, demonstrated strong FISH signals in heterochromatin regions [16].

Neotropical electric fish species, order Gymnotiformes, have shown their heterochromatin to be preferentially distributed in the pericentromeric regions of their chromosomes [13, 17, 18].

The investigation of repetitive sequences in *Gymnotus* seems promising for understanding the chromosomal evolutionary dynamics in the genus. DNA probes and chromosomal painting were applied to investigate chromosomal rearrangements in two species of the *G. carapo* complex, and rearrangements were found between the two species, involving several pairs of chromosomes, corroborating the existence of cryptic species in this group, in addition to the recent speciation between them [19, 20]. Analysis of the satellitome of some species by Next Generation Sequencing (NGS) revealed sets of conserved satellite sequences, but the CA, GA and GAG motifs when mapped revealed a useful band for identification of homologous chromosomes [21].

The ribosomal DNA mapping can also provide new answers about chromosomal evolution in the genus, and even serve as a tool for understanding geographic distribution patterns [22]. In the species that had the 18S rDNA mapped, the proposal of only one pair carrying the conserved nucleolus organizing regions (NORs) is plausible [17, 18, 23, 24]. Regarding the 5S rDNA, the group behaves as an attractive model for evolutionary studies, showing a species-specific pattern. The evolution of this gene family receives special attention in the species that comprise the group *G. carapo* with a diploid number of 54 chromosomes, which have from 14 to 19 pairs identified with this sequence [21]. This situation is totally different from the pattern observed for other species within the group and shared only by species carrying $2n = 54$ chromosomes.

2. Biogeography of electric fish and repetitive DNA sequences

The complex history of the formation of the South American rivers is fundamental to explain the diversity and distribution of aquatic biota in this region. Successive continental geomorphological changes, such as the one that resulted in the formation of the Andes, associated with historic and biological factors allow the identification of patterns that led to the formation of the largest and most diverse freshwater ichthyofauna on the planet [25, 26]. Such changes alter the drainage scenario forming lakes, capture headwaters and basins of varying sizes, or even isolate populations for certain periods, favoring the diversification of biota by vicariance and allopatric, in addition to promoting subsequent drainage coalescence, leading to enrichment and contact of organisms [26, 27].

The Gymnotiformes order comprises electric fish or knifefishes. Endemic to the Neotropical region [28], which are widely distributed, from the Pampas in Argentina to Chiapas, Mexico and reach their greatest diversity and abundance in the Amazon basin [29–31]. Members of the Gymnotiformes order, are unique in their ability to produce and recognize electrical signals, never left the South America plate, since their electrosensory system is not capable of functioning in brackish or salt water [21].

Regarding the karyotype, *Gymnotus* is also a diverse group, with a diploid number ranging from 34 chromosomes, verified in *G. capanema* [33] to 54, found in several species of the *G. carapo* group [18, 21, 23–34]. Sexual chromosomal heteromorphism has already been recorded in some, and all records follow a turnover model, with little heterochromatin and of recent origin [24, 35].

Among the *Gymnotus*, an intriguing fact has been reported for species that have $2n = 54$ chromosomes, present in the *carapo* Clade: *G. cf. carapo*, *G. inequilabiatus*, *G. paraguensis* and *G. mamiraua*. These species share the diploid number and also a large number of 5S rDNA sites [13], but they are geographically separated in different hydrographic basins. Though, *G. cf. carapo*, *G. inequilabiatus*, *G. paraguensis* occur in the Paraná-Paraguay basin, and *G. mamiraua* is distributed in the Amazon basin (**Figure 1**). The number of 5S DNA sites in *G. paraguensis*, *G. inaequilabiatus* and *G. cf. carapo* are 19, 17 and 15 bearing pairs, respectively [36–38], and 14 pairs in *G. mamiraua* [22].

For *G. paraguensis* and *G. mamiraua*, the 5S rDNA are present in the pericentromeric region and when sequenced, a transposition element similar to Tc1 was identified in the NTS (non-transcribed spacer), suggested as one of the mechanisms used for the transposition of this rDNA in chromosomes [22, 36]. In *G. inequilabiatus*, which has 17 sites of 5S rDNA, the sequencing of some clones with classes I and II revealed sequences of the TATA type in NTS, which are associated with the regulation of the expression of the 5S rDNA gene [37].

As a rule, studies investigating this ribosomal gene associate an increase in the number of 5S rDNA sites with the presence of pseudogenes, which would originate through duplication of copies, resulting from the transposition or duplication of genomic DNA, which would be facilitated by the organization tandem of this rDNA family. In addition, locating these sequences in the terminal portion of the

Figure 1. Location of species with $2n = 54$ chromosomes in the Amazon and Paraná-Paraguay basins: a) *G. mamiraua* in the Amazon basin; b) *G. carapo*; c) *G. paraguensis* and d) *G. inaequilabiatus*. The pairs with 5S rDNA are represented schematically along with each species.

chromosomes would facilitate the process of pseudogenization and the association with transposable elements [39].

Although the mechanism of origin and dispersion of these sequences in the $2n = 54$ *Gymnotus* genome is still unclear, it is possible to assume that the increase in the number of 5S-like rDNA sites and fixation in the genome accompanies the migratory dispersion of these species. In the case of *Gymnotus* ($2n = 54$), infection by this transposable element may have probably occurred in a common ancestor for species with $2n = 54$ chromosomes, before the final isolation between the Amazon and Paraná-Paraguay basins, since these species are currently found in different river basins. *Gymnotus mamiraua* was restricted to the Amazon basin show the smallest number of sites, 14 pairs, maybe the original satellite sequence like-5S rDNA; however, the isolated species in the Paraná-Paraguay basin experienced divergence from this satellite sequence, differing in number pairs with sequence, such as: *G. inaequilabiatus* (17 pairs), *G. paraguensis* (19 pairs), and *G. carapo* (15 pairs), (**Figure 2**) [13, 22].

Evidence of the connection between South American watersheds is reflected in the evolutionary history of the fish. Other species corroborate the hypothesis of interconnection between these two systems. Migratory species of the genus *Prochilodus* are considered as indicators of this panorama, where the relationship of sister clades of that genus has been useful for understanding the separation of the basins [Magdalena (Orinoco (Amazonas-Paraná)]. The use of molecular clock methodologies, with species in the group, estimates that the isolation between the Amazonas-Paraná basins must have occurred between 2.3–4.1 million years, with an estimated coalescence between strains of 1–3–3 million years [40]. Morphological and molecular analyzes with rheophilic taxa increase the evidence of connection between these basins [41]. The final rupture of the connection between the Paraná-Paraguay and Amazonas-Orinoco basins is inferred as a recent event, with the final uplift of the Andes that changed the course of these basins and isolated them [25, 42], with final separation estimates still uncertain and very peculiar to each group of fish investigated [43].

Figure 2. Topology obtained by the method of clustering neighbors (neighbor joining, NJ) for species of *Gymnotus* of the carapo clade, using the distance model Kimura-2-parameters (K2P) for the mitochondrial gene COI. The values present in the nodes are the bootstrap values (> 50%) calculated from 1000 replicates and the diploid number superimposed on the tree. The distribution of species is indicated on the map. * absence of coordinates, group not plotted on the map.

In order to understand the relationships between *Gymnotus*, here, an analysis of a 556 bp (base pairs) COI mitochondrial gene fragment was conducted in 21 specimens from the *carapo* Clade from the central Amazon basin and four more specimens from a population in the Munim River, MA. Sequences deposited in the NCBI database were added to this analysis, with the access numbers described in **Table 1**. The analysis of 51 sequences resulted in eight clades (**Figure 2**). The data revealed a

Species	Voucher	Locality	NCBI accession number
<i>G. sylvius</i>	LBP7069	Alto Paraná	GU.064995.1
<i>G. sylvius</i>	LBP31958	Alto Paraná	GU.701779.1
<i>G. sylvius</i>	LBP7070	Alto Paraná	GU.702209.1
<i>G. sylvius</i>	LBP8831	Alto Paraná	GU.701782.1
<i>G. sylvius</i>	LBP27380	Alto Paraná	GU.701778.1
<i>G. sylvius</i>	LBP29096	Alto Paraná	GU.702207.1
<i>G. sylvius</i>	LBP25852	Alto Paraná	GU.701758.1
<i>G. sylvius</i>	LBP31933	Alto Paraná	GU.701767.1
<i>G. sylvius</i>	LBP25853	Alto Paraná	GU.701762.1
<i>G. sylvius</i>	LBPV27382	Alto Paraná	JN.988881.1
<i>G. sylvius</i>	LBPV27381	Alto Paraná	JN.988880.1
<i>G. sylvius</i>	LBP9823	Alto Paraná	GU.701780.1
<i>G. pantanal</i>	MZUEL5644	Alto Paraná	KF.359492.1
<i>G. pantanal</i>	LBP31929	Alto Paraná	GU.701776.1
<i>G. pantanal</i>	LBP34742	Alto Paraná	GU.701775.1
<i>G. pantanal</i>	LBP34743	Alto Paraná	GU.701774.1
<i>G. pantanal</i>	LBP31927	Alto Paraná	GU.701773.1
<i>G. pantanal</i>	LBP31932	Alto Paraná	GU.701763.1
<i>G. carapo</i> Mara	GC7M	Bacia Costeira- Maranhão	XXXXXXXXXX
<i>G. carapo</i> Mara	GC9M	Bacia Costeira- Maranhão	XXXXXXXXXX
<i>G. carapo</i> Mara	GC11M	Bacia Costeira- Maranhão	XXXXXXXXXX
<i>G. carapo</i> Mara	GC18M	Bacia Costeira- Maranhão	XXXXXXXXXX
<i>G. carapo</i> Cat	GCAT11029	Amazônia Central	XXXXXXXXXX
<i>G. carapo</i> Cat	GCAT 11028	Amazônia Central	XXXXXXXXXX
<i>G. carapo</i> Cat	GCAT 11066	Amazônia Central	XXXXXXXXXX
<i>G. carapo</i> Cat	GCAT 11477	Amazonia Central	XXXXXXXXXX
<i>G. carapo</i>	GC2006	Peru Amazonas	KF533344
<i>G. carapo</i>	GC2007	Peru, Amazonas	KF533345.1
<i>G. mamiraua</i>	GM11078	Amazônia Central	XXXXXXXXXX
<i>G. mamiraua</i>	GM11733	Amazônia Central	XXXXXXXXXX
<i>G. mamiraua</i>	GM11731	Amazônia Central	XXXXXXXXXX
<i>G. mamiraua</i>	GM10947	Amazônia Central	XXXXXXXXXX
<i>G. mamiraua</i>	GM10948	Amazônia Central	XXXXXXXXXX
<i>G. mamiraua</i>	GM10949	Amazônia Central	XXXXXXXXXX
<i>G. mamiraua</i>	GM11730	Amazônia Central	XXXXXXXXXX

Species	Voucher	Locality	NCBI accession number
<i>G. inaequilabiatus</i>	MZUEL5649	Alto Paraná	KF.359490.1
<i>G. inaequilabiatus</i>	LBP26331	Alto Paraná	GU.701766.1
<i>G. inaequilabiatus</i>	LBP31931	Alto Paraná	GU.701764.1
<i>G. inaequilabiatus</i>	LBP25850	Alto Paraná	GU.701760.1
<i>G. inaequilabiatus</i>	LBP7071	Alto Paraná	GU.702210.1
<i>G. inaequilabiatus</i>	LBP29097	Alto Paraná	GU.702208.1
<i>G. inaequilabiatus</i>	LBP34744	Alto Paraná	GU.701781.1
<i>G. ucumara</i>	GU11575	Amazônia Central	XXXXXXXXXX
<i>G. ucumara</i>	GU11802	Amazônia Central	XXXXXXXXXX
<i>G. ucumara</i>	GU11701	Amazônia Central	XXXXXXXXXX
<i>G. ucumara</i>	GU11698	Amazônia Central	XXXXXXXXXX
<i>G. ucumara</i>	GU11574	Amazônia Central	XXXXXXXXXX

Table 1.
Access numbers in the NCBI of the *Gymnotus* species of the carapo clade used for genetic distance verification through DNA barcode.

high percentage for the distance between the species identified as *G. carapo* (**Table 1, Figure 2**). The sequences of individuals identified as *G. carapo* do not form exclusive groups. Three clades with high rates of genetic distance were observed between *G. carapo* “Catalão” (2n = 40, XX/XY) and *G. carapo* Maranhão (2n = 42), but between *G. carapo* “Catalão” and *G. carapo* do the distance between Amazonas and Peru was 0.02% (**Table 2**). The species *G. mamiraua* forms an exclusive clade with an average intraspecific genetic distance of 0.12% and *G. inaequilabiatus* the average was 0.2% (**Table 3**). These two species showed an interspecific distance of only 0.6%.

The results with the mitochondrial DNA COI obtained here validate the species of *carapo* Clade, with high rates of genetic distance between groups of species identified as *G. carapo*, confirming the probable existence of more than one taxonomic unit in this group. However, this did not occur for two species: *G. mamiraua* from the Amazon basin and *G. inaequilabiatus* from Alto Paraná, for which a low interspecific distance was detected and with a distance of less than 1% was detected, possibly causing recurrent speciation.

Cytogenetic data already pointed to the proximity between these two species, because in addition to sharing the same diploid number, both had many sites of

Intraspecific genetic distance	
Gp 1 - <i>G. syltvius</i>	0.013526931
Gp 2 - <i>G. Pantanal</i>	0
Gp 3 - <i>G. carapo</i> “Maranhão”	0.000893724
Gp 4 - <i>G. carapo</i> “Catalão”	0.000928937
Gp 5 - <i>G. carapo</i>	0.001858738
Gp 6 - <i>G. mamiraua</i>	0.001241471
Gp 7 - <i>G. inaequilabiatus</i>	0.002136221
Gp 8 - <i>G. ucumara</i>	0.003103677

Table 2.
Intraspecific genetic distance based on mutations of the COI gene, using the K-2-P model.

Interspecific diversity							
	Gp-1	Gp-2	Gp-3	Gp-4	Gp-5	Gp-6	Gp-7
Gp 1 <i>G. sylvius</i>							
Gp 2 <i>G. Pantanal</i>	0.062						
Gp 3 <i>G. carapo</i> “Maranhão”	0.168	0.156					
Gp 4 <i>G. carapo</i> “Catalão”	0.030	0.069	0.174				
Gp 5 <i>G. carapo</i>	0.028	0.071	0.171	0.002			
Gp 6 <i>G. mamiraua</i>	0.048	0.077	0.181	0.055	0.053		
Gp 7 <i>G. inaequilabiatus</i>	0.046	0.074	0.178	0.053	0.051	0.006	
Gp 8 <i>G. ucumara</i>	0.179	0.159	0.051	0.183	0.181	0.178	0.174

Table 3.
Matrix of the means of genetic distance (K2P) for the COI gene obtained among the different species of *Gymnotus* from carapo clade.

5S rDNA, a condition that is not common in fish. We suggest, by the results of diploid number and dispersion of 5S rDNA, that the species *G. paraguensis* and *G. carapo* (2n = 54) from Alto Paraná are also related to the last two already mentioned (**Figure 2**). Thus, what has been verified is that the 5S rDNA has been a potential tool in helping to reconstruct the steps involved in the evolution and biogeographic history of the species of the genus *Gymnotus*, especially the *carapo* Clade, inferred both by the chromosomal mapping and by the molecular analysis of that gene.

2.1 New repetitive sequences studies in the genus *Gymnotus*

The eukaryote genome is characterized by presenting nucleotide sequences with varied arrangements, generally forming two large groups, gene regions and repetitive DNA sequences. In fish of the genus *Gymnotus*, the latter has been associated with heterochromatin. The location of the heterochromatin is reported to be preferentially organized in the centromeric and pericentromeric regions [18, 19, 36, 44]. The prospection of repetitive sequences by the technique of DNA reassociation kinetics (C0t-1) proves to be a safe and fast technique for obtaining copies of highly and moderately repetitive DNA sequences [45]. Thus, it is possible to build libraries and screening repetitive DNAs, and has been used to isolate the highly repeated fraction of the plant genome [46] and animals [47] to significantly expand our knowledge of the organization of their chromosomes.

In the present study, repetitive DNA sequences were isolated by C0t-1 (**Figure 3**) and mapped in two species of electric fish, *G. paraguensis* and *G. sylvius*. Our objective was identifying the heterochromatin compositions and to verify the presence of repetitive sequences originated from transposable elements. Twelve specimens (four females and eight males) of *G. paraguensis* and 21 specimens (seven females and 14 males) of *G. sylvius* collected at Piquiri River, Paraná – Brazil were analyzed. The isolated probes from *G. paraguensis* had lengths of 473 and 206 bp (**Figure 4a**) and when submitted to BLAST (<http://www.ncbi.nlm.nih.gov/blast>), clone 2 was found to have 95% identity with microsatellites from *Salmo salar*. Dinucleotide repetitions were observed in this clone. The clones of *G. sylvius* had lengths of 124, 202 and 123 bp (**Figure 4b**). The results of hybridization with total C0t-1 in both species were coincident with heterochromatic sites, according to the description by C banding [18].

The heterochromatin of *Gymnotus* has been reported to be preferentially organized in the centromeric regions [18, 19], as detected in the present study

Figure 3. 1% agarose gel electrophoresis of DNA treated for kinetic re-association method (C_0t-1). a) after one minute in autoclave the DNA appears as a trail; b) after treatment with S1 nuclease enzyme the DNA appears with defined length between 100 and 400 bp. (methodology described in complementary material).

Figure 4. a) Alignment of two isolated clones of *G. paraguensis*, b) alignment of three clones of *G. sylvis*.

(**Figure 5a** and **b**), and in most species of the Gymnotidae family [36, 44]. Furthermore, heteromorphisms in length between homologous chromosomes of the NOR regions were observed in *G. paraguensis*, indicating differential accumulations of heterochromatin regions (**Figure 5b**). However, when the probes of one species were hybridized with the other (cross-FISH) no positive hybridization signals were observed (data not shown).

According to Charlesworth et al. [48] and Topp and Dave [49], the regions located nearest to the centromere show fast evolutionary rates due to low recombination, initiating the accumulation of repetitive DNA sequences, which explains its specificity. This association between heterochromatin and repetitive sequences is fundamental to the organization of important chromosomal structures such as the centromere. In a study with *Oreochromis niloticus* using the GISH (Genomic *in situ* Hybridization) methodology, the heterochromatin present in pericentromeric regions was found to be species-specific [50] and composed of repetitive and transposable elements [51–53].

Figure 5.
 Fluorescent in situ hybridization (FISH) performed with probes isolated for kinetic re-association of DNA (C_{0t-1}). a) *G. sylvius* karyotype; b) *G. paraguensis* karyotype. Observe in b the heteromorphism of nucleolus organizer regions in pair 25. Bar = 10 μ m.

The repetitive elements isolated from *G. sylvius* and *G. paraguensis* in the present study were located in the pericentromeric region, coincident with the heterochromatin observed by C banding. High levels of specificity of the isolated probes and of the species' genomes were assumed because no signals were observed in crossed FISH analyses - confirming the heterogeneous composition of the heterochromatin of these species.

Sequencing analyses showed exclusive sequences for both species, and although repetitive elements in the heterochromatin regions are present in distant eukaryotes groups such as *Drosophila* and plants [54, 55] as important structural regions of the genome, the structural functions of these sequences in *G. sylvius* and *G. paraguensis* are not yet known.

The Y chromosome of *Eigenmannia virescens* isolated by Henning et al. [56] had large amounts of heterochromatin and physical mapping with Y chromosome probe was performed with closely related species without differentiated sex chromosome systems, and the probes hybridized only in the centromeric and telomeric regions.

In addition to the two species analyzed in the present study, two other species of Gymnotiformes have had their repetitive DNA sequences analyzed and described. Claro [57] isolated the repetitive sequences of *G. sylvius* and *G. carapo* by enzymatic digestion with *AluI* and *HaeIII*. The isolated fragments with 300 bp showed dispersed distributions in both the species and similar locations using both enzymes. Furthermore, a transposable SINE element that labeled different regions in *G. carapo* was identified; all the sequences showed disperse labeling that was not coincident with heterochromatin regions, suggesting an important function in the evolution and organization of non-coding DNA regions [58].

More recently, the publication of Satellitome results has been awaited, a global study by NGS and bioinformatics of all satellite sequences of *Gymnotus* species from the main clades. According to previous data released by the authors, the massive characterization of satellite DNA families processes of genetic differentiation and the dynamics of these sequences among the representatives stand out [59], which corroborates our findings in the present work.

3. Conclusions

Molecular studies with the multigene family 5S rDNA in electric fish (Gymnotidae) have advanced a lot in recent years. The chromosomal location and distribution have been particularly interesting, since all species of the genus *Gymnotus*, analyzed so far, show great variability. Its distribution on the chromosomes of species with $2n = 54$ shows to be a biogeographic marker, suggesting that the speciation of this group was recent due to migratory expansion. On the other hand, the species analyzed in the present study showed repetitive sequences composed of microsatellite replications species-specific, present around the centromeres. Although the location of the heterochromatin in *Gymnotus* was conserved, they had different constitutions and could represent important evolutionary markers for cytotaxonomic studies of this group.

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Conflict of interest

The authors declare no conflict of interest.

A. Appendices

A.1 Methodology for obtaining cytochrome oxidase sequences

For the Cytochrome oxidase I gene, five specimens of *G. carapo* “Catalão”, four specimens of *G. carapo* “Maranhão”, four specimens of *G. ucumari* and seven specimens of *G. mamiraua* were sequenced, using the primers BOL-COIfishF1 (5'TCAACYAATCAYAAAGATATYGGCAC'3') and BOL-COI-fishR1 (5'-ACTTCYGGGTGRCCRAARAATCA- 3') [60]. Genomic DNA extraction was performed using the chloroform-phenol method [61]. The PCR reactions were performed in a final volume of 25 μ L, containing genomic DNA (100 ng), 10x buffer with 1.5 mM $MgCl_2$, Taq DNA polymerase (5 U/ μ L), dNTPs (1 mM), pair of primers (5 pM) and Milli-Q water. The conditions for amplification were: 1 min 95° C, followed by 30 cycles of 1 min at 94° C, 1 min at 59° C, 1 min 30 sec at 72° C and final extension of 5 min at 72° C.

Sequences of *Gymnotus* species for the Clado *carapo*, available in the NCBI database, from the Paraná-Paraguay basin were added to our analysis: *G. sylvius*, *G. inaequilabiatus*, *G. pantanal* and *G. carapo* “Pantanal” (Table 1).

The calculation of intra and interspecific distance was performed using the Mega 5 software [62] with the Kimura-2-parameters evolutionary model. The identification was carried out according to the protocol established by DNA barcoding through the Neighbor Joining (NJ) analysis [63], which consists of looking for the tree with the lowest total sum of branches, using Kimura-2-parameters as an evolutionary model (K2P) [64]. The topology confidence test was performed with

bootstrap analysis, containing 1000 replicates. Such analyzes were performed using the Mega 5 software [62].

The sequences were aligned in the program Clustal W [65], using the editor BioEdit 7.0 [66], were submitted to BLAST in the NCBI database (<http://www.ncbi.nlm.nih.gov>).

A.2 Methodology for obtaining cytogenetic material

Mitotic chromosomes were obtained according to the protocol described by Bertollo et al. [67]. Genomic DNA extraction was performed using the chloroform-phenol method [61]. Repetitive DNA probes were obtained using the C_{ot} -1 DNA technique as described by Zwick et al. [45] and adapted by Vicari et al. [68]. This technique is based on re-association kinetics and enzymatic digestion by S1 nuclease. The probes were labeled by nick translation with digoxigenin 11 dUTP (Roche®) and the signals were recognized by anti-digoxigenin-rhodamin (Roche®). The hybridization techniques followed the Pinkel et al. [69] protocol, with 77% of stringency (2.5 ng/ μ L of each probe, 50% of deionized formamide, 10% dextran sulphate, 2X SSC at 37°C for 18 hours). The chromosomes were counterstained with DAPI in Vectashield medium (Vector®). The chromosomal preparations were analyzed by epifluorescence microscopy using an Olympus BX41® microscope fitted with a CCD Olympus DP-71® digital camera. Image capture was performed using DP controller® software (Olympus). The probes with positive hybridization signals were purified and cloned using the pMOSBlue blunt ended RPN5110 cloning kit (Amersham Biociences®) for subsequent sequencing. The samples were sequenced in an ABI-PRISM 3100 Genetic Analyzer automatic sequencer at the ACTGene laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, RS).

The sequences were aligned and edited using the CLUSTAL W program [65] using the following parameters: weights 6.66 and 10.0 for opening and extension of gaps, respectively, for pairwise alignments, and weights 10.0 and 15.0 for opening and extension of gaps in the multiple alignments respectively. Clustering analysis was performed using the parsimony method on the PAUP program v. 4.0b10 [70]. The isolated fragments of repetitive DNA sequences (C_{ot} -1) from *G. sylvius* and *G. paraguensis* had lengths between 100 and 400 bp, respectively (**Figure 5**). These fragments were used as probes for fluorescent *in situ* hybridization (FISH) and were found to label most of the pericentromeric regions of the chromosomes of *G. sylvius* (**Figure 4a**) and all of the chromosomes of *G. paraguensis* (**Figure 4b**).

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